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Characterization of soluble and membrane-bound forms of a vanadate-sensitive ATPase from plasma membranes of the halotolerant alga *Dunaliella salina*

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A plasma-membrane preparation, obtained by an osmotic lysis of cells of the halotolerant alga *Dunaliella salina* (Sheffer and Avron, 1986) was further purified on glycerol gradients. Two fractions of a vanadate-sensitive ATPase activity were resolved on the gradients: a soluble and a membrane-bound fraction. The enzymes exhibit identical sensitivities to vanadate, dicyclohexylcarbodiimide, diethylstilbestrol, SH reagents and phloridzin but are insensitive to molybdate, nitrate, cyanide, azide and quercetin. The two ATPase activities also have an identical K_m value for Mg-ATP (0.9 mM), optimal activity at pH 7, are stimulated by 200 mM KCl or NaCl but inhibited by high salt concentrations. Antibodies against yeast plasma membrane H^+-ATPase cross-react with 92 kDa or with 60 kDa polypeptides in the plasma membrane and soluble ATPase fractions, respectively. Treatment of the purified plasma membranes with trypsin releases a soluble vanadate-sensitive ATPase from the membranes, and the release is protectable by ATP. It is suggested that the soluble ATPase, which is resolved on the glycerol gradients, is a proteolytic product of the plasma membrane ATPase which is a vanadate-sensitive H^+-ATPase.

Introduction

Vanadate-sensitive H^+-ATPases have been identified and purified from plasma membranes of a wide range of organisms including yeast, fungi and higher plants [1]. In contrast to mammalian cells it appears that in all cells which possess vanadate-sensitive H^+-ATPases it provides the major generator of the transmembrane electrical potential and pH gradients, and therefore, also the driving force for metabolite and ion transport across the cell membrane.

These enzymes are characterized by sensitivity to vanadate, diethylstilbestrol (DES), dicyclohexylcarbodiimide (DCCD), SH reagents, by a high specificity for ATP, by formation of a phosphorylated intermediate and by being built of one major polypeptide of about 100 kDa.

The primary sequence of the enzyme from yeast and fungi has been determined and it contains several highly conserved stretches with other cation translocating ATPases [1].

Recently it was reported that a vanadate-sensitive ATPase activity exists also in membrane fractions isolated from the halotolerant alga *Dunaliella* [2]. A unique physiological quality of this alga is that it can adapt itself to an extremely wide range of NaCl concentrations (0.1–5 M) by denovo synthesis of intracellular glycerol at concentrations which osmotically balance the extracellular NaCl [3]. We have observed that inhibitors of PM H^+-ATPase such as vanadate, DES and DCCD inhibit K^+ uptake, Na^+ extrusion and the recovery of *D. salina* from hyperosmotic shocks indicating that this enzyme plays a major role in ionic and osmotic regulation in *Dunaliella* [4,5].

Recently a method to prepare plasma membrane vesicles from *D. salina* by a mild osmotic lysis was developed in our laboratory [6,7]. In this work we describe a further purification of this membrane preparation which is based on the low permeability of

Abbreviations: PM, plasma membranes; DCCD, dicyclohexylcarbodiimide; DES, diethylstylbestrol; PCMBS, *p*-chloromercurybenzenesulphonate; NEM, *N*-ethylmaleimide; Mops, 4-morpholinepropanesulphonic acid; SDS, sodium dodecylsulphate; PAGE, polyacrylamide gel electrophoresis, PBS, phosphate buffer saline; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Mes, 4-morpholineethanesulphonic acid.

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these membranes to glycerol, and characterize a vanadate-sensitive ATPase which is present in this preparation. An unusual phenomenon which we observed is that throughout the purification a major part of the activity is released from the membrane in a soluble form, probably as a result of a proteolytic modification.

Materials and Methods

Preparation of plasma membrane vesicles from Dunaliella salina

D. salina cells were cultured in media containing 1 M NaCl, and plasma membrane vesicles were isolated from osmotically lysed cells by a modification of a previously described procedure [6,7]. $(4–8) \cdot 10^{10}$ cells in late logarithmic phase were collected by centrifugation, washed once in 1 M NaCl, 10 mM Tris-Mops (pH 7), 2 mM $MgCl_2$ and once in 1.6 M glycerol, 10 mM Tris-Mops (pH 7), 10 mM NaCl, 2 mM $MgCl_2$. Cells were resuspended in the latter medium to about 10^9 cells/ml and mixed for 30 min at 0°C with 4 volumes of the same medium lacking glycerol and containing 1 mM benzamidine and 5 mM ξ-amino caproic acid. Chloroplasts, nuclei and unbroken cells were removed by centrifugation at $12\,000 \times g$ for 20 min, and the microsomal fraction was collected by centrifugation at $150\,000 \times g$ for 60 min. The pellet was suspended and hand homogenized in the above medium containing 0.4 M glycerol (3–5 ml) and recentrifuged at $2500 \times g$ for 5 min to remove residual thylakoid membranes. The supernatant was diluted to 25 ml with the same medium and centrifuged again at $150\,000 \times g$ for 60 min, the pellet was resuspended in about 2 ml of the same medium, homogenized, and recentrifuged as above to remove residual thylakoids. This preparation is referred to as 'crude PM'. Samples of crude PM containing 2–3 mg protein were applied to 7–60% glycerol gradients (10 ml) containing also 10 mM Tris-Mops (pH 7), 30 mM KCl, 1 mM $MgCl_2$, 1 mM benzamidine and 5 mM ξ-aminocaproic acid and centrifuged for 2 h at $35\,000 \times g$ in SW-41 rotors at 2°C. The pellet, containing the purified plasma membranes was resuspended in about 1 ml of 0.4 M glycerol medium, homogenized, centrifuged at $2500 \times g$ for 10 min and the supernatant was stored at liquid nitrogen (purified PM).

ATPase assay

Enzyme samples (5–40 μg protein) were incubated in 200 μl containing 20 mM Tris-Mops (pH 7), 50 mM KCl, 8 mM $MgCl_2$ and 2 mM γ-^{32}P-ATP containing 10–20 μCi γ-^{32}P/mmol ATP for 30 min at 37°C. The reaction was stopped by addition of 0.5 ml ice-cold trichloroacetic acid (7%), the protein was removed by centrifugation (microfuge). ^{32}P was extracted as a phosphomolybdate complex in isobutanolbenzene two-phase system as previously described [8].

Analytical procedures

Protein was determined by a modification of the Lowry procedure [9]. Separation of proteins on 5–15% or on 7–17% polyacrilamide SDS gels was according to Laemmli [10]. Bloting to nitrocellulose filters was done according to Gershoni et al. [11]. The blot was incubated in a solution containing phosphate buffer saline (PBS), 10% skimmed milk and 0.05 triton X-100 for 1 h at 22°C. The blot was next incubated with rabbit antisera against purified *Saccharomyces cerevisiae* H^+-ATPase (a gift from Prof. N. Nelson, Nutley, NJ), diluted 200-fold in the blotting solution for 14 h at 4°C. The blot was next washed 3 times in the blotting solution. Detection of bound antibodies was performed with affinity purified goat anti-rabbit antibodies conjugated to horseradish peroxidase diluted 1000-fold in blotting solution. Color development was performed by immersing the blot in PBS solution containing 0.025% diaminobenzidine and 0.03% H_2O_2.

Results

Characterization of a vanadate-sensitive ATPase in a plasma membrane preparation from Dunaliella salina

A method to isolate plasma membranes from *Dunaliella* by a mild hypotonic shock and differential centrifugation has been developed recently in our laboratory [6,7]. The plasma membrane origin of this preparation was confirmed by labeling cells prior to their lysis with an impermeable fluorescent marker and by the unusual lipid composition of this preparation [6,12].

This preparation possesses an Mg-ATPase activity which is sensitive to vanadate, dicyclohexylcarbodiimide (DCCD), diethylstilbestrol (DES) phloridzin and different SH reagents (Hg^{2+}, PCMBS, NEM) which are characteristic inhibitors of plasma membranes (PM) H^+-ATPases in plants, yeast and fungi (Table I). The ATPase activity is insensitive to molybdate, a phosphatase inhibitor, to nitrate, a vacuolar ATPase inhibitor and to azide, cyanide and quercetin-inhibitors of mitochondrial and chloroplast coupling factors.

The affinity for vanadate is influenced both by ionic composition and by ligands of the enzyme: K_i (vanadate) increases from 1 μM in the absence of salt to 4.5 μM in 200 mM KCl, while phosphate and ATP protect against vanadate inhibition (data not shown). A typical property of PM H^+-ATPase from plants is the partial stimulation by K^+ and by other monovalent cations. As shown in Fig. 1, 200 mM KCl as well as NaCl partially stimulate (20–30%) while higher salt concentrations strongly inhibit the vanadate-sensitive ATPase activity of the *D. salina* PM preparation. The pH dependence of the activity (Fig. 2, upper trace) reveals biphasic optima – one at pH 9, which is essentially insensitive to vanadate, and another around pH 7. These results suggest

TABLE I

Characterization of the inhibitor-sensitivity of D. salina PM ATPase

Crude PM vesicles (30 μg protein) were preincubated with the indicated inhibitors for 10 min at 0 °C, or for 15 min at 22 °C at pH 6 (with DCCD only), before the ATPase assay. K_{50} is the inhibitor concentration required for 50% inhibition.

Inhibitor	Concentration	ATPase activity (%)	K_{50}
Vanadate	50 μM	20	1.2 μM
DCCD	100 μM	23	20 μM
DES	100 μM	16	30 μM
Phloridzin	10 mM	17	3 mM
Quercetin	300 μM	105	–
NaF	5 mM	40	4 mM
NaN_3	5 mM	89	–
$NaNO_3$	50 mM	92	–
$(NH_4)MoO_4$	100 μM	93	–
Quercetin	300 μM	105	–
KCN	5 mM	115	–
$HgCl_2$	100 μM	3	30 μM
PCMBS	100 μM	25	55 μM
NEM	10 mM	26	3 mM

that the preparation contains a PM H^+-ATPase, but may be contaminated with other ATP-hydrolases.

Purification of D. salina plasma membranes on glycerol gradients

A partial purification of *D. salina* PM was achieved previously on a sucrose gradient [6]. In an attempt to improve this purification we chose a glycerol gradient instead of a sucrose gradient, the rational being that the plasma membrane is supposed to be impermeable to glycerol [13] and therefore should selectively shrink in high glycerol on the gradient, and consequently increase its apparent density. An additional low-speed centrifugation step, which removes residual chloroplast and mitochondrial contaminations was also introduced.

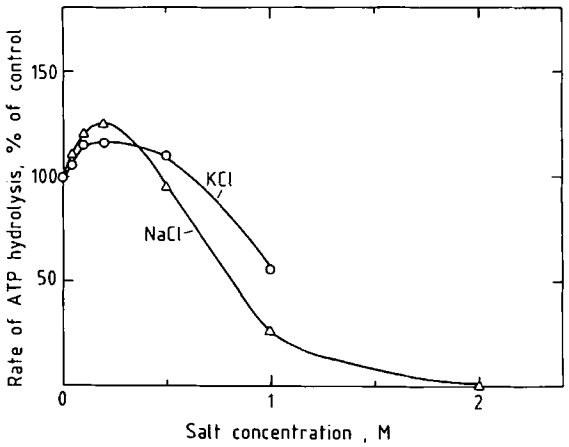

Fig. 1. Effect of salt on ATP hydrolysis catalysed by *D. salina* PM ATPase. ATPase activity of *D. salina* crude PM was assayed in the presence of the indicated NaCl or KCl concentrations.

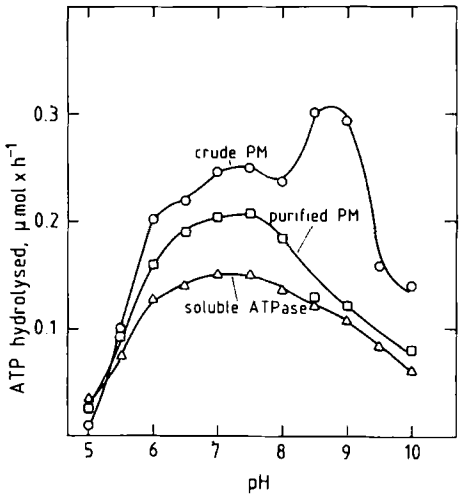

Fig. 2. pH dependence for ATP hydrolysis of different ATPase preparations from *D. salina*. Samples of crude PM (40 μg protein), purified PM (25 μg protein) or soluble ATPase (12 μg protein) were assayed for ATPase activity in the presence of the following buffers: Tris-Mes (pH 5.0–6.5), Tris-Hepes (pH 7.0–9.0) or Tris-glycine (pH 9.5–10.0).

Analysis of ATPase activity and protein distribution of PM separated on a 7–60% glycerol gradient is demonstrated in Fig. 3 and in Table II. Two ATPase activity peaks are observed: a diffuse band (10–20% glycerol) and a membranal heavy band at the pellet. The major protein peak, banding at 35% glycerol, turned out to represent pure ribosomes. This was confirmed by equilibrium sedimentation on an analytical ultracentrifuge, by the 260 nm/280 nm absorption ratio of 1, by in vivo ^{32}P incorporation and by sensitivity to RNAase, proteases and Mg depletion (EDTA medium, data not shown). Also the polypeptide profile (Fig. 4, lane 1) is characteristic to plant ribosomal proteins.

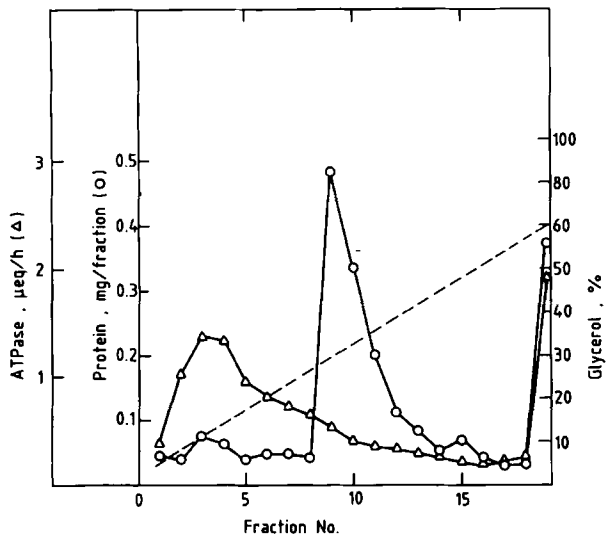

Fig. 3. Protein and ATP distribution on glycerol gradients. Crude PM preparation (2.2 mg protein) was separated on 7–60% glycerol gradients (2 h at 35000 r.p.m.), fractionated (0.6 ml fractions) and analyzed for protein and ATPase activity.

TABLE II

Purification of a vanadate-sensitive ATPase from D. salina PM

10 l of *D. salina* cells ($3 \cdot 10^{10}$ cells) were harvested and the plasma membrane vesicles were prepared as described under Materials and Methods.

Preparation	Protein		ATPase activity		
	total (mg)	recovery (%)	total (μmol per h)	recovery (%)	specific activity (μmol per mg protein per h)
Crude PM	2.5	(100)	4.25	(100)	1.7
Purified PM	0.38	15	1.60	37	4.2
Soluble ATPase	0.20	8	2.40	56	12.0

In order to check whether the two ATPase fractions contain membranes we cultured cells in ^{14}C-bicarbonate in the light for several days to obtain uniform labeling, and analyzed the glycerol gradient fractions for lipid content by extraction with chloroform-methanol [14].

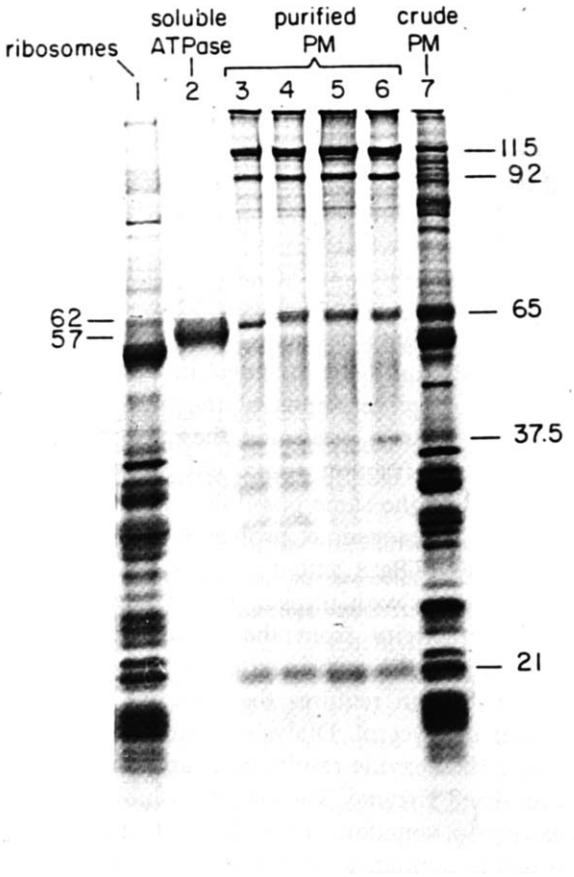

Fig. 4. SDS-PAGE analysis of polypeptide compositions of fractions from glycerol gradient separation of *D. salina* plasma membranes. Different fractions from glycerol gradient purification were separated on 7–17% SDS-polyacrylamide gels and stained with coomassie blue. (1) Ribosomes (Fr. 9, Fig. 3), 35 μg protein, (2) soluble ATPase (Fr. 3, Fig. 3), 15 μg; (3–6) purified PM from different preparations (Fr. 20, Fig. 3), 20–30 μg protein; (7) crude PM preparation before purification, 40 μg protein.

TABLE III

Protein and lipid content analysis of glycerol gradient analyzed by ^{14}C labeling

D. salina cells (1.7 l, $3 \cdot 10^6$ cells/ml) were preincubated with 10 mM sodium bicarbonate containing 0.5 mCi ^{14}C-bicarbonate in a sealed flask for 48 h in the light, and washed twice before preparation of plasma membranes. Lipids were extracted from chloroform/methanol/water mixtures (1 : 1 : 0.9) as described elsewhere [14]. ^{14}C recovery is expressed as a percentage from total counts in the crude PM before separation on glycerol gradients.

Preparation	^{14}C recovery (% of total)		Lipid content (% ^{14}C)
	protein	lipid	
Purified PM	10	4.8	32.4
Soluble ATPase	8.6	0.2	2.0
Ribosomes	56	0.5	0.8

As is demonstrated in Table III only the heavy ATPase fraction contained a significant amount of lipids, indicating that the lighter ATPase fraction is a soluble enzyme. Analysis on PAGE (Fig. 4) reveals that the membranal ATPase preparation (lanes 3–7) contains five major polypeptide bands (with apparent molecular masses of 115, 92, 65, 37.5 and 21 kDa) and several minor bands whereas the soluble ATPase preparation (lane 2) contains one or two major bands of M_r around 60 000.

A comparison of the inhibitor sensitivity of the two preparations (Table IV) demonstrates an essentially identical sensitivity to vanadate, DES, DCCD, phloridzin and PCMBS, lack of inhibition by nitrate, molibdate and azide and the biphasic effect of NaCl.

The pH dependence of the soluble and membranal ATPase preparation (Fig. 2) is also similar. Mg-ATP is the best substrate for both enzymes with $K_m = 0.9$ mM (calculated at 10 mM Mg and varying ATP). Ca^{2+} and

TABLE IV

Comparison of inhibitor-sensitive of PM, soluble and trypsin-solubilized ATPase from D. saliina

Purified PM (15 μg) and soluble (7 μg) ATPase preparations were preincubated with inhibitors and analyzed as in Table I.

Inhibitor		ATPase activity (% of control)		
		purified PM	soluble	trypsin-solubilized
Vanadate	1 μM	65	62	53
DES	100 μM	35	22	30
DCCD	100 μM	17	35	–
Phloridzin	5 mM	37	39	50
PCMPS	300 μM	16	10	12
Sodium nitrate	50 mM	75	112	–
$(NH_4)_2$-molybdate	0.2 mM	75	90	–
NaN_3	5 mM	82	87	–
NaCl	200 mM	125	147	–
	1 M	26	65	–

Mn^{2+} partially stimulate ATP hydrolysis (85% and 73%, respectively, of Mg-ATPase activity at 10 mM). Co^{2+} and Ni^{2+} have no effect, and Zn^{2+}, Cd^{2+} and Ln^{3+} strongly inhibit the Mg-ATPase activity (70%, 88% and 95% inhibition, respectively, at 10 mM).

In an attempt to identify the ATPase polypeptides in the membrane and soluble ATPase fractions, the polypeptides of both fractions were separated by polyacrylamide gel electrophoresis blotted to nitrocellulose filters, and incubated with antibodies raised against purified yeast plasma membrane H^+-ATPase. The rabbit antibodies were identified with peroxidase-conjugated goat anti-rabbit antibodies. The analysis reveals two different polypeptids which cross-react with the anti-yeast H^+-ATPase antibodies – a 92 kDa polypeptide which appears to be a minor polypeptide component of purified plasma membranes (Fig. 5, lane 3) and a 60 kDa polypeptide in the soluble ATPase fraction (Fig. 5, lane 4).

These results indicate that the soluble and the membranal preparations contain the same ATPase, which is very likely a plasma membrane H^+-ATPase.

Is the soluble ATPase a proteolytic product of the PM-ATPase?

A possible reason for the existence of a soluble and a membranal form of the ATPase is proteolitic cleavage which may cause a partial release of the enzyme from the membrane. In order to try to prevent putative proteases we have included different protease inhibitors or excess of exogenous protein (bovine serum albumin) during the preparation. Two of the protease inhibitors – benzamidin and ξ-amino caproic acid – significantly increased the activity of the ATPase preparations and therefore were routinely included in all subsequently preparations. However, none of the protease inhibitor or the added protein prevented the appearance of the soluble ATPase fraction (data not shown). Another approach to test the same possibility was to expose the purified PM to exogenous proteases in an attempt to solubilize the ATPase activity. As is demonstrated in Fig. 6 incubation with trypsin indeed releases most of the ATPase activity from the membrane, and ATP protects against the trypsin effect. The release of the enzyme by trypsin requires the presence of high concentrations of glycerol. Dialysis of the glycerol solution containing the enzyme results in a rapid inactivation of the solubilized enzyme. The trypsin-solubilized ATPase is sensitive to vanadate, DES, DCCD, phloridzin and SH reagents similar to the PM and soluble ATPase fractions (Table IV). Analysis of the trypsin-released proteins by PAGE revealed three major bands having molecular masses of about 60, 40 and 20 kDa (not shown). These results suggest that the soluble ATPase preparation which is resolved in the glycerol gradient may be a proteolitic product of the PM-ATPase which is released from the membrane during the purification.

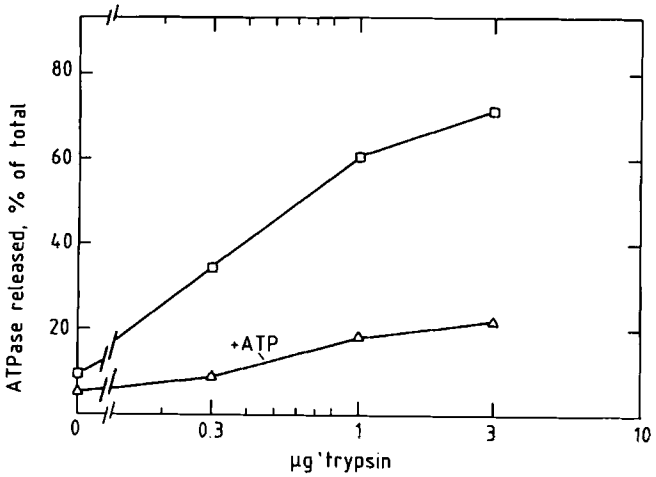

Fig. 6. Release of ATPase from purified plasma membranes of *D. salina* by trypsin. Purified *D. salina* PM (50 μg) were incubated for 30 min at 37 °C with the indicated amounts of trypsin in 250 μl containing also 10% glycerol and 20 mM Tris-Mops (pH 7) with or without 10 mM ATP. The reaction was stopped on ice with 10 μg trypsin inhibitor, the membranes were removed by centrifugation (in a Beckman airfuge, 15 min at 120000 × *g* and the supernatants were analysed for ATPase activity. Activity is expressed as a percentage of total released ATPase activity.

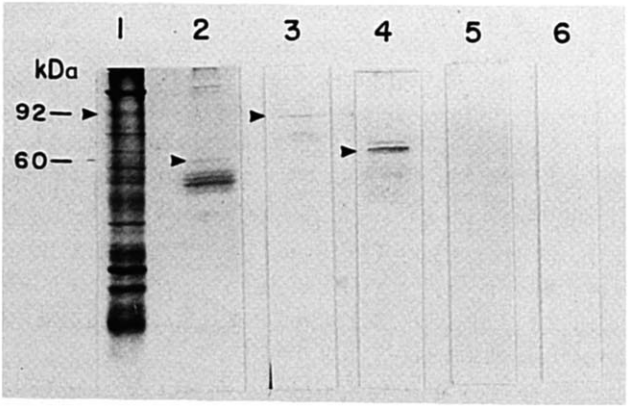

Fig. 5. Cross-reaction between yeast H^+-ATPase antibodies and proteins in soluble and plasma membrane ATPase preparation. Plasma membranes and soluble ATPase were purified as in Fig. 3 except that EDTA (0.5 mM) was added to the glycerol gradient instead of Mg. Samples of plasma membranes (40 μg protein) and soluble ATPase (15 μg protein) were separated on 7.5–15% SDS-PAGE, stained with coomassie blue (lanes 1 and 2) or transferred to nitrocellulose sheets and immunodecorated with rabbit antisera against purified *S. cerevisae* H^+-ATPase (lanes 3 and 4) or control rabbit serum (lanes 5 and 6) and treated with peroxidase conjugated goat-anti-rabbit antibodies. The crossreactive polypeptides are marked by arrows on the blots and stained lanes, and their molecular masses are indicated. (1) Stained PM; (2) stained soluble ATPase; (3) peroxidase, PM treated with H^+-ATPase antibodies; (4) peroxidase, soluble ATPase treated with H^+-ATPase antibodies; (5) peroxidase, PM treated with control serum; (6) peroxidase, soluble ATPase treated with control serum.

Discussion

The purification of the PM preparation from *D. salina* is based on the unusually high apparent density of this membranes in the presence of glycerol. The possibility of contamination by other membrane vesicles of intracellular origin seems a priori quite unlikely due to this unusually high density. Nevertheless, the presence of ribosomes in the crude membrane preparation raises the possibility of contamination by endoplasmic reticulum and possibly also by other membranes. In order to check the purity of the preparation we have analyzed the purified PM by different density gradients (sucrose, ficoll and percoll), by gel filtration, and by two-phase partition but non of these methods resolved more than one membrane population. The preparation is free of chlorophyll, cytochromes or other pigments and is insensitive to ATPase inhibitors of vacuolar (nitrate) mitochondrial (azide) membranes suggesting that the preparation is pure. The origin of the ribosomes in the crude preparation is not clear. One possible reason is that the ribosomes are released during the lysis of the cells and absorb to the plasma membrane. The dissociation of intact ribosomes from the PM in the presence of Mg^{2+} on the glycerol gradient is consistent with this possibility. Another protein which sometimes appears as a contamination in the PM preparation and also dissociates from the membrane on the glycerol gradients is the chloroplast ribulose diphosphate carboxylase (50 and 15 kDa polypeptides). It appears, therefore, that *Dunaliella* ribosomes are particularly 'sticky', perhaps as a consequence of adaptation to function in high glycerol which is the natural intracellular osmoregulant in the alga. Five polypeptide bands (of apparent molecular masses 115, 92, 65, 37 and 21 kDa) are highly enriched in the purified PM preparation (Fig. 4, lanes 3–6) and therefore appear to be integral PM proteins.

The group of Gimmler has also described three ATPase activities in *D. parva* cells broken by Yeda press system [2]. One of these activities, suggested to be plasma membrane vanadate-sensitive ATPase, exhibits similar properties to the presently described enzyme; notably it is stimulated by Ca^{2+} as well as by Mg^{2+} as by salts, an effect which was found to be due to anion stimulation [15].

Although we still do not have a direct evidence that the *D. salina* vanadate-sensitive ATPase activity is a PM H^+-ATPase, we have presented a lot of circumstantial evidence which suggests that this is indeed the case.

(1) The sensitivity to vanadate, diethylstilbestrol, DCCD and SH reagents is characteristic to PM H^+-ATPases from other origins [1]. Similarly, we demonstrated sensitivity to phloridzin, a known ATPase inhibitor. We also found that the ATPase activity is sensitive to erytrosin and to 2,2,2-trichloroethyl-3,4-dichlorocarbamilate which are considered specific PM H^+-ATPase inhibitors [1,16], but is unaffected by fusiccocin, which stimulates the activity of PM H^+-ATPase from plants.

(2) The substrate specificity and affinity for Mg-ATP resembles that of purified PM H^+ ATPases from other origins. The only apparent difference is the partial activation of ATP hydrolysis by Ca^{2+}, which usually inhibits H^+-ATPase from other sources [17].

(3) The partial stimulation of ATP hydrolysis by KCl is characteristic to PM H^+-ATPase [17].

(4) The requirement for glycerol as a stabilizer, both for the soluble ATPase, which is released on the glycerol gradient, and for detergent solubilized enzyme (not shown) was found also for other PM H^+-ATPases [17].

(5) Preliminary studies with PM vesicles prepared from *D. acidophila* cultured at pH 1 show that it contains 10–50 times more vanadate-sensitive ATPase activity and catalyses ATP-dependent H^+ uptake (Sekler, I., Glasser, U. and Pick, U., unpublished results). It is conceivable that an acidophile will overproduce a PM H^+-ATPase to cope with the large uphill pH gradient.

At present it is not clear why we cannot detect ATP-dependent H^+ uptake in *D. salina* PM vesicles. It seems possible that a relatively high permeability to protons combined with a low concentration of enzyme in our vesicle preparation results in formation of small pH gradients which are below our detection sensitivity. This possibility seems likely in light of the low ATP-hydrolyzing activity in our membrane preparation. Nevertheless, the possibility that the ATPase is a different kind of ion pump should not be completely discounted.

The release of a soluble ATPase from the plasma membrane is surprising in view of the fact that all other known PM H^+-ATPases, as well as the closely related mammalian Na,K-ATPase and Ca-ATPase, are integral membrane proteins which contain several hydrophobic stretches that probably span the membrane and they can be solubilized only by detergents [1].

The observation that trypsin treatment releases the ATPase from purified PM may suggest that it detaches a hydrophobic fragment from the enzyme which loosens its association with the membrane. At present it is not clear whether the appearance of the enzyme in a soluble form is a natural phenomenon in *D. salina*,, reflecting for instance unprocessed or a partially degraded turnover product, or whether it is an accidental consequence of our preparation procedure. It is possible, for example, that the osmotic lysis of the cells activates an endogenous protease which leads to cleavage of the ATPase. To check such a possibility we are now trying to purify PM from *D. salina* by using alternative procedures which do not involve osmotic lysis.

Nevertheless, irrespective of its origin, the availabil-

ity of a soluble and catalytically active ATPase preparation provides an important experimental system for structural and functional analysis for example by crystalization and X-ray analysis.

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